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PRINCIPAL INVESTIGATOR(S): Richard A. Cerione, Ph.D.

CONTRACTING ORGANIZATION: Cornell University
Ithaca, New York 14853-6401

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Richard A. Cerione, Ph.D.

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Cornell University
Ithaca, New York 14853-6401

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In this report, we describe our progress during the past year in identifying the steps underlying the activation of the neu/erbB2 tyrosine kinase, a protein felt to be an early participant in the development of breast cancer. During the past year we have shown that the growth factor, heregulin, stimulates the formation of an erbB3-neu/erbB2 heterodimeric receptor complex in intact cells and that this leads to the phosphorylation of erbB3. We also have found that other types of receptor heterodimer complexes are possible, including EGF receptor-erbB3 and EGF receptor-neu/erbB2 interactions. In addition, we have identified two novel proteins that may represent downstream components of the signaling pathways of the EGF receptor and neu/erbB2.

In the coming year, we plan to expand on this work and characterize heregulin-erbB3 and erbB3-neu/erbB2 interactions in mammary carcinoma cells in detail. We also will determine the functional outcome of EGF receptor-neu/erbB2 heterodimer formation. In addition, we will determine the involvement of a newly identified 130 kDa phosphosubstrate in the signaling pathway initiated by the EGF receptor and the involvement of a novel nuclear, heregulin-stimulated GTP-binding protein in the growth of mammary cells. Finally, we will explore the utility of animal model systems for testing reagents that will be generated as an outcome of our molecular studies.

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Principal Investigator's Signature

6/13/95
Date

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INTRODUCTION

The studies outlined in the original proposal were aimed at determining the molecular basis underlying the actions of the neu/erbB2 tyrosine kinase and its involvement in the development and progression of breast cancer. Within this section of the annual report (for the period July 15, 1994-June 15, 1995), I will discuss the background of the research problem (within the context of previous work performed by our laboratory and other laboratories), the purpose of the present research and the methods of approach that are being used.

Background of the research problem- It is well accepted that cancer is the outcome of a loss of regulation at one or more points in a growth factor-coupled signal transduction pathway. In many cases of breast cancer, the activation of a growth factor receptor first called neu (1), and then more recently erbB2 (2) or HER2 (for the human protein), may represent the first step in an aberrant signaling pathway that is responsible for this disease. The neu/erbB2 tyrosine kinase is a member of a large superfamily of transmembrane receptors. Members of this superfamily that share the highest degree of sequence similarity with neu/erbB2 make up the subclass 1 receptor tyrosine kinases and include the epidermal growth factor (EGF) receptor (3,4), the erbB3 (HER3) protein (5,6), and the erbB4 (HER4) protein (7). Each of these receptors is comprised of a single polypeptide chain (M_r ~ 170-180 kDa) that includes a cysteine-rich extracellular (growth factor binding) domain, a single transmembranal helix of 23 amino acids, and a cytoplasmic tyrosine kinase domain that contains a number of potential autophosphorylation sites.

Much of the focus of the proposed studies is to understand the molecular regulation of the neu/erbB2 tyrosine kinase, with a particular emphasis on the mechanisms underlying its activation. The oncogenic capability of the *neu* gene was identified in chemically-induced rat neuroblastomas (1). A single point mutation that resulted in the substitution of a glutamic acid for a valine residue at position 664 within the transmembrane domain of the neu tyrosine kinase was shown to result in oncogenic activation. Thus far, no mutation analogous to that which is responsible for the oncogenicity of the rat protein has been detected in the human counterpart of neu (i.e. the erbB2 or HER2 proteins). However, given the correlation between the levels of erbB2 and the poor prognosis for breast cancer patients, it has been assumed that high expression of erbB2 causes an over-stimulation of a signaling pathway that causes the proliferation of mammary cells.

Because of the apparent importance of the regulation of the neu/erbB2 tyrosine kinase activity, a great deal of effort has been directed toward the isolation and identification of a ligand/growth factor for this receptor. It originally was reported that a family of glycoproteins called the heregulins (also referred to as the neu-differentiation-factor or NDF) served as ligands for neu/erbB2 in breast cancer cells (8-11). Both NDF and the heregulins were shown to stimulate tyrosine phosphorylation in breast cancer cells. However, not all cells that express neu/erbB2 bind heregulin or NDF (e.g. fibroblasts). Thus, breast cancer cells must possess a component that enables neu/erbB2 to respond to heregulin. We have found that the same holds true for rat pheochromocytoma (PC12) cells which undergo neurite extension in response to the activation of the neu/erbB2 receptor (see below). Given the tendency of members of the EGF receptor family to form heterodimers, it was tempting to speculate that another member of the family (i.e. aside from neu/erbB2) initially binds heregulin (or NDF) and then forms a heterodimer with neu/erbB2 and confers (to neu/erbB2) responsiveness to heregulin.

Using insect cell-expressed tyrosine kinases, we found that erbB3, which is ~60% identical to the EGF receptor and neu/erbB2, served as a receptor for heregulin (12). Work by Plowman and colleagues similarly showed that erbB4 also is capable of serving as a receptor for heregulin (13). It is interesting, however, that while erbB4 is capable of autophoshorylation and tyrosine kinase activity in a heregulin-stimulated manner, erbB3 is not. We have shown that erbB3, itself, is capable of little or no tyrosine kinase activity (14), either in the presence or absence of added heregulin, probably because erbB3 differs from all other kinases at four positions (in particular there is an asparagine residue at position 834 which is an aspartic acid in all other kinases and is thought to be an essential residue in the active center of the cyclic AMP-dependent protein kinase). However, transfection of cells with erbB3 and neu/erbB2 reconstitutes a higher affinity heregulin

receptor (K_d ~ 50 pM compared to a K_d of ~ 1 nM for erbB3 alone) and a heregulin-stimulated phosphorylation of both the neu/erbB2 and erbB3 proteins (15; also see Gamett et al., in press, Appendix I). Taken together, these results have led us to propose that heregulin binding to erbB3 promotes the formation of an erbB3-neu/erbB2 heterodimer which enables the cross-phosphorylation of erbB3 by neu/erbB2. In this regard, we also have suggested that erbB3 may represent a new class of receptor molecules that provides the potential for dual regulation in growth factor signaling, specifically, by enabling other receptors (i.e. neu/erbB2) to respond to unique ligands (heregulins) and by serving as a target for cross-phosphorylation reactions that elicit the recruitment of specific substrates. More recently, we have shown that activated (transforming) neu/erbB2 was able to induce neurite extension in PC12 cells in a manner analogous to the nerve growth factor (NGF) receptor (trk) [16] and that heregulin will stimulate this response in PC12 cells expressing normal neu/erbB2. Thus, we suspected that in PC12 cells, erbB3 or a related protein component, acting in conjunction with neu/erbB2, was probably responsible for conferring responsiveness to heregulin (see below).

Purpose of the present research/Experimental Methods- The overall goal of the studies outlined in the original proposal was to understand the molecular basis underlying the actions of neu/erbB2 and heregulin in different cells (e.g. PC12 cells, breast cancer cells) and to demonstrate that heregulin interactions with erbB3 may have important implications in the development of breast cancer. Based on the studies outlined above, we have developed the following hypothesis regarding the molecular mechanisms for the activation of the neu/erbB2 tyrosine kinase. The binding of heregulin to the erbB3 receptor protein stimulates the formation of a heterodimer between erbB3 and the neu/erbB2 tyrosine kinase. This enables heregulin to bind with high affinity and to stimulate the tyrosine kinase activity of neu/erbB2. Because of the presence of a number of candidate (tyrosine) phosphorylation sites within the carboxyl terminal domain of erbB3, and because these tyrosine residues when phosphorylated would be predicted to bind tightly to the SH2 domains of other candidate signaling molecules, we also have proposed that erbB3 serves as an adapter molecule in the neu/erbB2 signaling pathway. The heregulin-stimulated phosphorylation of erbB3, within a ternary complex consisting of heregulin-erbB3-neu/erbB2, would serve to recruit the next protein(s) in the signaling cascade. We originally proposed that one such signaling protein was the src kinase because it had been reported to play a role in the development of some breast cancers (17) and because phosphorylated erbB3 was predicted to form a tight complex with the SH2 domain of src (18). Recently, we have found that another SH2 domain-containing protein, the 85 kDa regulatory subunit of the PI 3-kinase, forms a specific complex with erbB3 in a heregulin-dependent manner in PC12 cells (Gamett et al., in press, see below). Thus, we envisage signaling cascades that are initiated by the binding of heregulin to erbB3 and then proceed through the formation of erbB3-neu/erbB2 heterodimers, which lead to the phosphorylation of erbB3 (by neu/erbB2) and the recruitment and/or activation of the src kinase or the PI 3-kinase.

The studies that are being supported by our DOD grant focus on verifying the occurrence of these signaling cascades in neuronal cells, where neu/erbB2 map play a key role in development, and in mammary cells where neu/erbB2 may be involved in the regulation of cell growth and in the development of breast cancer. We feel that the identification and biochemical characterization of protein components that comprise the neu/erbB2 signaling cascade will provide potential therapeutic targets for countering the development of breast cancer. To do this, we are using a combination of protein biochemistry and recombinant DNA technology to dissect the neu/erbB2 signaling pathway. Through cDNA transfection approaches, we are introducing the cDNAs for wild type and mutant neu/erbB2 and erbB3 into cells to determine the potential for interaction between these proteins in a heregulin-dependent manner. These studies also depend heavily on specific antibody reagents against the neu/erbB2 and erbB3 proteins that are now available in the laboratory. These antibodies are used in immunoprecipitation and Western blotting experiments to verify the existence of different signaling complexes containing the neu/erbB2 and erbB3 proteins. We also are using *E. coli* and *Spodoptera frugiperda* expression systems to generate recombinant fusion proteins that can be used as affinity reagents to isolate signaling partners and to generate

recombinant proteins (e.g. insect cell-expressed neu/erbB2 and erbB3) for detailed biochemical characterization. The ultimate goal will be to utilize the information that we gain from biochemical and molecular biology-based studies in the development of therapeutic strategies.

BODY/"PROGRESS REPORT'

The following is a progress report regarding the work that has been performed by our laboratory over the past year as funded by the Department of Defense Breast Cancer Initiative USAMRDC grant DAMD17-94-J-4123. This report is subdivided into the following sections, i.) Description of objectives, ii.) Progress of research, iii.) Manuscripts resulting from the research, iv.) Personnel involved in the studies, and v.) Meetings where the research will be presented.

Description of objectives- The original proposal submitted to the Department of Defense Breast Cancer Initiative was largely based on our findings that the neu/erbB2 protein is not a receptor for heregulin, as originally thought, but rather the related erbB3 protein serves as a receptor for heregulin and thus may play an essential role in conferring responsiveness of neu/erbB2 to heregulin (through the formation of erbB3-neu/erbB2 heterodimers). This led us to hypothesize that signal transduction as initiated by a heregulin-activated neu/erbB2 tyrosine kinase may proceed in the following manner. Heregulin first binds to erbB3; this binding event promotes the formation of an erbB3-neu/erbB2 heterodimer which stimulates the tyrosine kinase activity of neu/erbB2 and leads to the (cross)phosphorylation of erbB3. The phosphorylated erbB3 protein is then able to recruit other cellular proteins (primarily those containing SH2 domains) and thereby initiate signaling cascades which when integrated have a positive effect on mammary cell growth. Given this hypothesis, we proposed to verify that the cellular actions of heregulin were in fact mediated by its initial binding to the erbB3 protein. We also wanted to show that heregulin-stimulated erbB3-neu/erbB2 interactions occurred in intact cells. Once these points were verified, we intended to characterize heregulin-erbB3 binding interactions with the longer range goal of developing reagents (e.g. erbB3 antibodies, erbB3-extracellular domain) that might interfere with these interactions and thus influence neu/erbB2 signaling and potentially serve as therapeutic agents. We also hoped to determine what happens after the heregulin-stimulated erbB3-neu/erbB2 interaction, i.e. identify the other cellular proteins that act in the signaling pathway. While these various aims were being pursued, we also intended to determine whether the EGF receptor might play an important role in signaling pathways relevant to the development of mammary carcinomas, for example, through the EGF-stimulated activation of neu/erbB2. Along these lines, it seemed attractive to contemplate the possibility that both EGF and heregulin (through distinct mechanisms) could positively impact on neu/erbB2 activity. Finally, the long range goal of the proposal was to use the information generated from these molecular studies to begin to design possible therapeutic strategies. Specifically, we hoped to determine that spontaneous mammary carcinomas that occur in the dog would serve as a suitable model for human breast cancer.

During the past year, we have made substantial progress toward achieving many of the objectives outlined above. We have documented the occurrence of heregulin-stimulated erbB3-neu/erbB2 interactions within cells and we have shown that these interactions lead to the tyrosine phosphorylation of erbB3 at specific sites and to the formation of a complex between tyrosine phosphorylated erbB3 and the 85 kDa regulatory subunit of the phosphatidylinositol 3-kinase. We also have shown the occurrence of EGF receptor-erbB3 and EGF receptor-neu/erbB2 interactions in intact cells. While the EGF-stimulated phosphorylation of neu/erbB2 does not result in the activation of the neu/erbB2 tyrosine kinase activity, our preliminary results suggest that an outcome of an EGF-stimulated interaction between the EGF receptor and neu/erbB2 in cells is a stimulated neu/erbB2 tyrosine kinase activity (perhaps by virtue of another tyrosine kinase joining the EGF receptor-neu/erbB2 complex, e.g. src). Finally, during the past year, we have identified two potentially new signaling participants. One is a 130 kDa protein that is highly phosphorylated by the EGF receptor in mammary carcinoma cells. The other is an 18 kDa GTP-binding protein that appears to be exclusively nuclear localized and whose GTP-binding activity is strongly stimulated

by heregulin (presumably through erbB3-neu/erbB2 interactions). All of these findings will be summarized in more detail below.

Progress of research:

1. Demonstration of erbB3-neu/erbB2 interactions in intact cells- As outlined above, a major reason for studying the molecular basis underlying the activation of the neu/erbB2 tyrosine kinase are the implications that neu/erbB2 participates in the development of human cancers (in particular, human breast and cervical cancers). Interestingly, it also has been suspected that neu/erbB2 plays a role in neuronal development since a putative ligand/growth factor for neu/erbB2, called heregulin or the neu differentiation factor (NDF), is identical to glial growth factor (19) and acetylcholine receptor-inducing activity [ARIA (20)]. Along these lines, we have shown that the expression of a transforming version of the neu/erbB2 tyrosine kinase (i.e. where the valine residue at position 664 within the transmembranal domain has been changed to a glutamic acid) is capable of stimulating neurite extension in rat pheochromocytoma (PC12) cells (16). Moreover, we found that the addition of heregulin to PC12 cells that over-express the normal neu/erbB2 tyrosine kinase also elicits neurite extension. These results thus highlighted two important points. The first was that the activation of neu/erbB2, either by a point mutation that increases its tyrosine kinase activity or by the addition of its putative ligand/growth factor, elicits a cellular morphology that is virtually identical to that induced by the nerve growth factor (NGF) receptor (i.e. trk) but distinct from the cellular effects elicited by the EGF receptor. Thus, PC12 cells offer an excellent model system for distinguishing important features of the signaling pathways initiated by activated neu/erbB2 versus the activated EGF receptor. A second major implication is that the addition of heregulin leads to the activation of the neu/erbB2 tyrosine kinase in PC12 cells. Thus, like breast cancer cells, PC12 cells must contain the necessary components for the stimulatory regulation of neu/erbB2 by heregulin.

Given these important features of PC12 cells, we have used these cells as a model system to address two important issues. The first was to determine if the erbB3 protein participated with neu/erbB2 in eliciting heregulin-stimulated neurite extension in PC12 cells. The second was to identify differences in the signaling cascades initiated by heregulin and EGF.

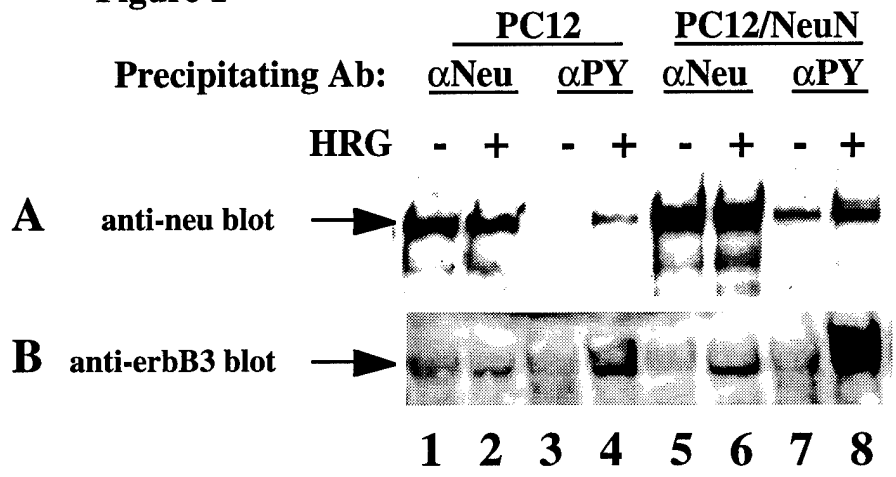
We first found that heregulin stimulates the tyrosine phosphorylation of endogenous erbB3 protein in PC12 cells as visualized by immunoprecipitating erbB3 with a specific monoclonal antibody and then Western blotting with anti-phosphotyrosine (compare lanes 3 and 4 in panel B of Figure 1, below, also see Gamett et al., in press; Appendix I). This phosphorylation, as well as a heregulin-stimulated phosphorylation of neu/erbB2 (which appears as a doublet), is greatly enhanced in cells over-expressing neu/erbB2 (see lanes 7 and 8 in panels A and B in Figure 1). The erbB3 phospho-band was broad and appeared to contain a component with lesser electrophoretic mobility than that found in the anti-neu/erbB2 precipitates. This will be considered further below. It also is important to note that while the erbB4 protein (another putative receptor for heregulin) could be detected by Western blotting anti-phosphotyrosine precipitates with a specific anti-erbB4 antibody, the tyrosine phosphorylation of erbB4 was not stimulated by heregulin in PC12 cells. We also found that over-expressing the erbB3 protein in PC12 cells led to heregulin-stimulated tyrosine phosphorylation of neu/erbB2 and erbB3 (to greater extents than observed in parental PC12 cells) and a heregulin-stimulated neurite extension (Gamett et al., in press), similar to the phenotypes obtained upon heregulin addition to cells over-expressing the normal neu/erbB2 tyrosine kinase or when transforming neu/erbB2 was expressed in PC12 cells.

Two important questions concerned whether the neu/erbB2 and erbB3 proteins actually formed a complex in PC12 cells and if this complex formation was stimulated by heregulin. The results of immunoprecipitation experiments using an anti-neu antibody indicated that the addition of heregulin either to PC12 cells that over-express neu/erbB2 (see lanes 3 and 4 in panel B of Figure 1), or PC12 cells that over-express erbB3 (Gamett et al., in press; Appendix I), led to the precipitation of the neu/erbB2 and erbB3 proteins. Thus, overall, these results were consistent with a scheme where heregulin-stimulated heterodimer formation between neu/erbB2 and erbB3 results in increased tyrosine phosphorylation of the neu/erbB2 and erbB3 proteins and accounts for the ability of PC12 cells to respond to this growth factor. However, the fact that substantially

more of the heregulin-stimulated erbB3-neu/erbB2 complex was found in cells over-expressing neu/erbB2 or erbB3 compared to the parental PC12 cells suggests that the affinity of neu/erbB2 for erbB3 is relatively weak, such that sufficient complex formation can only be detected by immunoprecipitation under conditions where the amount of one or the other of these proteins is relatively high.

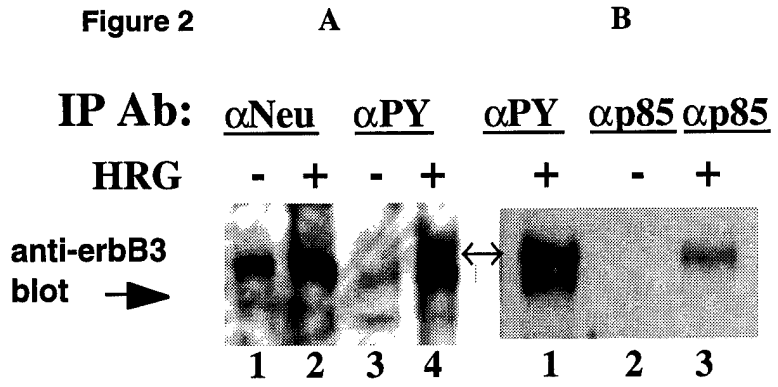
Since various lines of evidence (21) have suggested that one of the primary effector/targets for phosphorylated erbB3 is the 85 kDa regulatory subunit (p85) of the PI 3-kinase, we examined whether heregulin addition to PC12 cells might lead to the formation of a stable erbB3-p85 complex. In addition, given that we had evidence for the existence of two or more phosphorylated forms of erbB3 in heregulin-treated PC12 cells, while only a single erbB3 phospho-band appeared to associate with neu/erbB2, we were interested in determining whether there were distinct roles for the different (phospho)forms of erbB3. Immunoprecipitation experiments using a specific (precipitating) anti-p85 antibody, followed by Western blotting with a specific anti-erbB3 antibody, provided evidence for a direct interaction between these proteins. This interaction was most evident in PC12 cells over-expressing neu/erbB2 and was heregulin-stimulated. When comparing the results of anti-phosphotyrosine immunoprecipitates with those of anti-p85 immunoprecipitates (where in both cases the resuspended precipitates were blotted with anti-erbB3 antibody), we found that the erbB3 band that co-precipitated with p85 was identical in mobility to the slowest mobility erbB3 band detected in anti-phosphotyrosine precipitates (see the arrow in Figure 2, below). However, this slow-mobility erbB3 band was not detected in anti-neu/erbB2

Figure 1

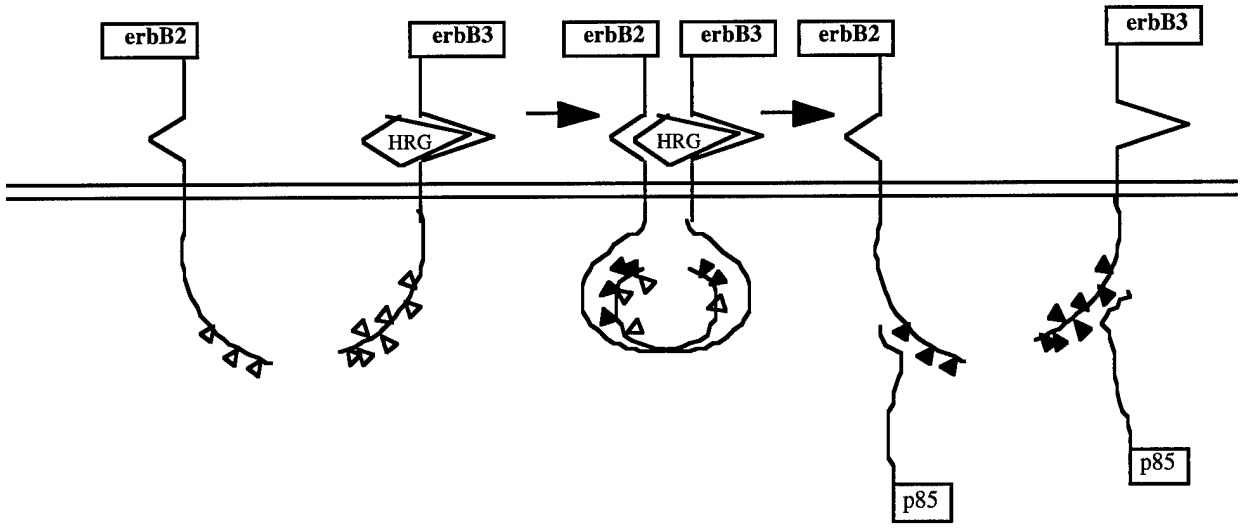


immunoprecipitates. These results suggested that the erbB3 protein is phosphorylated at multiple tyrosine residues in a heregulin- and neu/erbB2-dependent manner and that one of these phosphorylated erbB3 species forms a stable complex with neu/erbB2 but not with p85.

Figure 2



These results appear to be consistent with the simple scheme shown below. The binding of



heregulin (HRG) to the erbB3 protein stimulates the formation of an erbB3-neu/erbB2 heterodimer. This results in the increased tyrosine phosphorylation of neu/erbB2, at multiple sites (shown by the triangles), and the cross-phosphorylation of multiple tyrosine residues on erbB3. At the present time, it is not clear how heregulin stimulates the tyrosine phosphorylation of the neu/erbB2 protein. While one obvious possibility would have been the cross-phosphorylation of neu/erbB2 by erbB3, this seems unlikely because erbB3 shows little or no tyrosine kinase activity (14). However, it cannot be ruled out that within an erbB3-neu/erbB2 heterodimer, erbB3 is still able to cross-phosphorylate neu/erbB2, despite its weak kinase activity, because of the immediate proximity of the substrate (neu/erbB2). An alternative possibility is that another tyrosine kinase (e.g. src) joins the neu/erbB2-erbB3 complex and phosphorylates neu/erbB2 and a third possibility is that protein-protein interactions between neu/erbB2 and erbB3 result in an activation of neu/erbB2 tyrosine kinase activity. Whatever the mechanism, once activated, neu/erbB2 should be able to effectively cross-phosphorylate erbB3. The timing of this cross-phosphorylation event is critical, since the extent of tyrosine phosphorylation of erbB3 determines whether erbB3 remains complexed with neu/erbB2 or forms a new complex with p85. These findings then raise the interesting possibility that the heregulin-stimulated cross-phosphorylation of erbB3, by neu/erbB2, leads to the dissociation of erbB3 from neu/erbB2 and results in the specific binding of erbB3 to a

potential target, p85. In this regard, the mechanism would be similar to those commonly proposed for hormone receptor/G protein-mediated signaling cascades where the G protein first becomes activated within a hormone-receptor-G protein complex and then the activated G protein dissociates from this receptor and seeks out its target/effector molecule.

2. Interactions of the EGF receptor with other members of subclass 1- It is interesting that we have found that the signaling cascade involving erbB3 and p85 in PC12 cells (described in the preceding section) appears to be specifically initiated by heregulin and not by EGF. Both heregulin and EGF stimulate the tyrosine phosphorylation of erbB3 with the stimulatory effects of heregulin, but not EGF, being greatly enhanced in PC12 cells that over-express neu/erbB2. The electrophoretic mobilities of the erbB3 bands are clearly different for heregulin and EGF treatment (see Gamett et al., in press; Appendix I). The heregulin-stimulated PC12 cells show the two forms of erbB3 described in the preceding section. The faster mobility erbB3 phospho-band, stimulated by heregulin treatment, co-migrates with the slower mobility EGF-stimulated erbB3 phospho-band. Thus, both the EGF receptor and neu/erbB2 can elicit a common phosphorylation event within the erbB3 protein and this phosphorylated erbB3 species (when heregulin-stimulated) can remain complexed with neu/erbB2. There also is an erbB3 phospho-band detected in EGF-treated PC12 cells that has a greater mobility than any of the phospho-bands detected in heregulin-treated cells. While the addition of EGF to PC12 cells over-expressing neu/erbB2 leads both to the tyrosine phosphorylation of neu/erbB2 and erbB3, neither of these phosphorylated proteins appears to form a stable complex with p85.

An important question concerns the functional outcome of EGF receptor-neu/erbB2 interactions. Various studies have reported that the EGF receptor and neu/erbB2 will physically associate within cells and that this complex formation leads to cross-phosphorylation (22,23). However, the functional consequence of this interaction has not been determined. Recently, we have used a well defined *in vitro* system to specifically examine the functional outcome of neu/erbB2 cross-phosphorylation by the EGF receptor (Guy et al., submitted; Appendix I). We used purified plasma membranes from human epidermoid carcinoma (A431) cells as a source of EGF receptor because this preparation is highly active (when assayed for tyrosine kinase activity) and the EGF receptor is the tyrosine phosphorylated protein detected in these membranes. The neu/erbB2 protein used in these experiments was the insect cell-expressed 85 kDa soluble neu/erbB2 tyrosine kinase domain that contains the entire intracellular portion of the rat neu/erbB2 receptor except for 12 amino acid residues in the juxtamembrane region (this is designated from here on as NTK). The NTK was partially purified from the soluble fraction of recombinant virus-infected Sf9 cells by gel filtration on a Bio Gel A-0.5 column and was the only detectable phospho-protein in the preparation after the addition of ATP (24).

NTK, alone, was capable of an autophosphorylation reaction that yielded a maximum stoichiometry of incorporation of ~1 mol phosphate per mol NTK. In the presence of the EGF-stimulated EGF receptor, the final extent of tyrosine phosphorylation of NTK was doubled (to ~2 mol $^{32}P_i$ incorporated per mol of NTK). These results suggest that the EGF receptor phosphorylates NTK at a residue that is inaccessible to autophosphorylation. We then found that under conditions where the autophosphorylation of NTK was weak and barely detectable, while the EGF receptor-catalyzed tyrosine phosphorylation of NTK was strong, there was no significant difference in the ability of NTK to phosphorylate either a tyrosine-containing peptide (i.e. E_4Y_1, a repeating polymer of glutamic acid and tyrosine) nor a protein phospho-substrate, the 85 kDa regulatory subunit (p85) of the PI 3-kinase. These results suggest that the EGF receptor-catalyzed cross-phosphorylation of neu/erbB2 does not stimulate neu/erbB2 kinase activity toward exogenous substrates.

One clear outcome of the EGF receptor-catalyzed cross-phosphorylation of NTK was enhanced ability of NTK to bind to SH2-domain containing proteins (i.e. src and p85). The enhanced binding of NTK to a GST-p85 protein was not translated into an enhanced ability of NTK to phosphorylate p85. Thus, it is possible that the enhancement in the binding of NTK to p85 has another purpose, i.e. to recruit p85 (or a related SH2 domain-containing protein) to the membrane. It is interesting that this type of signaling mechanism does not appear to be bi-

directional. Specifically, we have not detected any ability of the recombinant neu/erbB2 tyrosine kinase domain to phosphorylate the EGF receptor nor to significantly promote the ability of the EGF receptor to associate with an SH2 domain-containing protein, i.e. p85. Moreover, we have not detected a heregulin-stimulated phosporylation of the EGF receptor in rat PC12 cells under conditions where an EGF-stimulated tyrosine phosphorylation of neu/erbB2 was observed (Gamett and Cerione, data not shown).

It also is interesting that our preliminary data suggests that the formation of an EGF receptor-neu/erbB2 complex in PC12 cells results in the activation of the neu/erbB2 tyrosine kinase. These results would differ from those that we have obtained using the *in vitro* system to characterize EGF receptor-neu/erbB2 interactions. Specifically, we have found that when neu/erbB2 was precipitated from EGF-treated cells, it had a higher tyrosine kinase activity toward the synthetic peptide substrate, E_4Y_1, compared to neu/erbB2 precipitated from non-EGF-treated cells. These results then suggest either that the EGF receptor promotes the interaction of another tyrosine kinase (e.g. src) with neu/erbB2, which then results in neu/erbB2 activation, or that the EGF-stimulated formation of a heterodimer between the full length EGF receptor and full length neu/erbB2 results in the activation of neu/erbB2 tyrosine kinase activity. The latter possibility would have interesting implications because it would suggest that growth factor binding to different receptors (i.e. EGF binding to the EGF receptor or heregulin binding to erbB3) could promote the formation of heterodimers that contain neu/erbB2 and result in the activation of neu/erbB2 tyrosine kinase activity. During the coming year, we will set out to distinguish between these possibilities.

3. Identification of a novel ~130 kDa phospho-substrate for the EGF receptor in mammary carcinoma cells- Because we had proposed that the src tyrosine kinase might be an important participant in growth factor-coupled signaling pathways in mammary carcinoma cells, we first set out to determine whether the src kinase might be capable of forming a stable complex with erbB3 in an EGF-stimulated manner. Thus far, these experiments have yielded negative results. However, during the course of these studies, we identified a 130 kDa protein (designated from here on as p130) that appeared to be the major tyrosine phospho-substrate for the EGF receptor in breast cancer cells (MDA-MB-468 cells and SKBR3 cells)(Flanders et al., in preparation; also, Flanders et al., Oncogene meeting abstract; Appendix II). It was interesting that p130 was not phosphorylated in a heregulin-dependent manner, either in mammary cells or in PC12 cells (although a p130-like protein was tyrosine phosphorylated in an EGF-dependent manner in PC12 cells). The p130 protein showed a mobility on various SDS-gel systems that was identical to a 130 kDa phospho-substratre for v-src that had previously been identified (25). This v-src substrate has been designated Cas, for Crk-associated substrate, because it forms a stable complex with the Crk oncogene product. Similarly, we have found that p130 will form a complex with Crk and that this complex shows immunoreactivity against a monoclonal antibody raised against Cas. However, recently we have found that the picture is somewhat more complicated. Specifically, the abilities of p130 and Cas to be precipitated by different SH3 domains are not identical. We have found that while the SH3 domain of src, when expressed as a GST fusion protein, very effectively precipitates tyrosine phosphorylated Cas, the SH3 domain of Grb2 does not. On the other hand, the GST-Grb2-SH3 domain effectively precipitates p130. Thus, we feel that p130 is distinct from Cas, although it is possible that p130 exists in a complex with Cas and c-src. At the present time, our working hypothesis is that p130 binds to the EGF receptor via Grb2 (i.e. as an outcome of an interaction between p130 and the SH3 domain of Grb2). p130 is then tyrosine phosphorylated and recruits Crk, or a complex of Crk and src. This may lead to the generation of distinct signaling pathways through the Crk and src proteins. In the future, we hope to clone p130 from a mammary cell cDNA library and to establish an expression system for recombinant p130 in *E. coli* or in insect cells in order to ultimately reconstitute the interactions of the purified recombinant protein with the purified EGF receptor.

4. Identification of a novel heregulin-stimulated GTP-binding protein- A long term goal of our laboratory has been to determine whether GTP-binding proteins act as molecular

switches in the nucleus, given that there are a number of nuclear activities where such regulation would seem beneficial including nuclear import and export, assembly-disassembly of the nuclear envelope and nuclear pore during the cell cycle, and the assembly of transcriptional regulatory complexes. Recently, we have discovered a GTP-binding activity in PC12 cells that is strongly regulated by growth factors (Wilson et al., Abstract for FASEB meeting on Ras GTP-binding proteins; Appendix II). The GTP-binding activity of this 18 kDa protein (designated from here on as p18), as read-out by light-activated incorporation of [α^{32}P]GTP, is stimulated by prior treatment of the cells with heregulin and nerve growth factor (NGF), but not by treatment with EGF. In this regard, it is interesting that heregulin like NGF stimulates neurite extension in PC12 cells (Gamett et al., in press), whereas EGF does not, suggesting that there may be some connection between the abilities of these growth factors to promote the GTP-binding activity of this nuclear protein and their ability to stimulate a specific morphological response. Roughly equivalent amounts of p18 appear to be present in the nuclear envelope and in the (soluble) nuclear contents. It also appears that p18 will associate with the 25 kDa nuclear GTP-binding protein, Ran, which has been implicated in nuclear import and in the regulation of various aspects of cell-cycle-progression (26). The association between p18 and Ran has been visualized by examining the ability of recombinant GST-Ran fusion proteins (either in the guanine nucleotide-depleted state, or in the GDP-bound or GTPγS-bound states) to precipitate p18 upon addition of glutathione agarose, The association of p18 with Ran occurs best when Ran is in the guanine nucleotide-depleted state. Interestingly, it is this form of Ran that binds best to its guanine nucleotide exchange factor (GEF), the RCC1 protein (27), which stimulates the exchange of GTP for GDP on Ran and has been shown to bind directly to DNA and to influence chromosomal condensation. We have shown that p18 also can be precipitated from cells with a specific anti-RCC1 monoclonal antibody. Thus, taken together, these results suggest that p18 forms a complex with Ran and RCC1. A the present time, we do not feel that growth factor (heregulin, NGF) treatment promotes the formation of this ternary complex, but rather causes the activation of a nucleotide exchange factor (possibly RCC1) that then stimulates GDP dissociation from p18 and promotes its binding to cellular GTP.

We are currently attempting to purify sufficient amounts of p18 from PC12 cells for micro-sequencing and ultimately for molecular cloning efforts. In the future, we also intend to determine how growth factor binding to the cell surface influences the GTP-binding activity of p18 in the nucleus, and in particular, determine if the growth factor regulation occurs through a Ras/Map kinase pathway or through a distinct signaling pathway. Recently, we have demonstrated that heregulin-stimulates the GTP-binding activity of an 18 kDa protein within nuclear fractions from human mammary carcinoma (SKBR3) cells. It will be interesting to determine whether similar heregulin-stimulated signaling pathways, that lead to the nucleus, are operating in PC12 cells and in mammary cells and establish why these pathways are not responsive to EGF.

CONCLUSIONS

Significance of the Research- The EGF receptor, neu/erbB2 and other members of the subgroup 1 growth factor receptors have been the major focus of the studies outlined in this proposal because the different members of this family have been implicated in human cancers. The amplification of the EGF receptor has been found in squamous cell carcinomas, bladder, lung, and liver cancers, and in gliomas (28,29). Over-expressed neu/erbB2 has been found in a significant proportion of human breast cancers (and hence its focus in these studies) (30-36) and erbB3 also has been suggested to be associated with human malignancy (5). In addition, members of this receptor family have been implicated in neuronal development and differentiation. This is especially the case for neu/erbB2 and erbB3 because the growth factor, heregulin, which promotes the formation of neu/erbB2-erbB3 heterodimers and stimulates the activation of neu/erbB2 activity, is identical to glial growth factor (19) and to the acetylcholine receptor-inducing activity (ARIA) (20), and because activated neu/erbB2 causes neurite extension in PC12 cells (16).

The overall goal of the proposed studies was to understand how neu/erbB2 becomes activated by heregulin and what other cellular proteins might play a role in heregulin or neu/erbB2-

initiated signaling pathways. During the past year, we have shown that heregulin stimulates the formation of erbB3-neu/erbB2 complexes in intact cells and that this leads to the tyrosine phosphorylation of erbB3. In PC12 cells, where heregulin stimulated signaling is coupled to neurite extension, we also found the formation of a specific interaction between tyrosine phosphorylated erbB3 and the regulatory subunit of the PI 3-kinase activity. We hypothesize that this interaction represents an important early step in heregulin-coupled signal transduction. We also have shown that other types of receptor heterodimer complexes are possible, including EGF receptor-erbB3 and EGF receptor-neu/erbB2 interactions; the latter appears to represent an alternative pathway (i.e. in addition to heregulin) for the activation of neu/erbB2. In addition to these findings, we also have discovered two novel proteins that may represent 'downstream components' in the signaling pathways of the EGF receptor and neu/erbB2. One of these is an 130 kDa cytosolic protein which appears to be a specific participant in EGF receptor signaling in mammary carcinoma cells and may serve to interface the EGF receptor with the Crk oncogene product and with the src tyrosine kinase. The second novel protein is an 18 kDa GTP-binding protein that is present in the nucleus and may play a key role in translating signaling events at the plasma membrane and cytosol into specific nuclear activities. By further delineating the molecular mechanisms underlying the interactions of the EGF receptor with the 130 kDa cytosolic phospho-substrate, and the interactions of the neu/erbB2 tyrosine kinase with the PI 3-kinase and the 18 kDa GTP-binding protein, we hope to identify novel signaling interactions that might provide new possibilities for therapeutic intervention against breast cancer.

Methods of approach: Plans for the coming year- In the coming year, we plan to expand on the work that has been performed by our laboratory (outlined above in the "Progress Report") and characterize heregulin-erbB3 and erbB3-neu/erbB2 interactions in both PC12 cells and in mammary carcinoma cells in detail. We also will determine the functional outcome of EGF receptor-erbB3 and EGF receptor-neu/erbB2 heterodimer formation. The importance of erbB3-PI 3-kinase interactions in mammary carcinoma cells also will be examined as will the importance of neu/erbB2-erbB3-src and EGF receptor-erbB3-src interactions. In addition, we will determine the involvement of a novel nuclear, heregulin-stimulated GTP binding protein in the growth of mammary cells. Finally, a longer term goal will be to take advantage of the facilities and expertise available at the College of Veterinary Medicine at Cornell to explore the utility of animal model systems for testing reagents that will be generated as an outcome of the results of our molecular studies.

These research goals will be addressed through the following specific aims.

1.) ***Examination of heregulin-stimulated erbB3-p85 interactions in mammary carcinoma cells***- During the past year, we found that EGF and heregulin stimulate the tyrosine phosphorylation of erbB3 at specific (distinct) sites in PC12 cells. The heregulin-stimulated phosphorylation of erbB3 (but not the EGF-stimulated phosphorylation) results in the formation of a stable complex between erbB3 and p85. We are in the process of determining whether the formation of this (heregulin-stimulated) erbB3-p85 complex is correlated with the ability of heregulin to stimulate PI 3-kinase activity in PC12 cells and if this is correlated with the specific ability of heregulin to stimulate neurite extension. We then will determine whether EGF and heregulin are able to stimulate specific tyrosine phosphorylations of erbB3 in a variety of human breast cancer cells that we have available in the laboratory (i.e. MDA-MB-453 cells that show heregulin-stimulated tyrosine phosphorylation and cross-linking to neu/erbB2 and MD-MB-468 cells that do not appear to contain neu/erbB2). These experiments will be performed through the immunoprecipitation of neu/erbB2- and erbB3-complexes using specific anti-neu and anti-erbB3 antibodies that are available in the laboratory (see Gamett et al., in press). As a complementary line of approach to the studies in intact cells, we also will set out to reconstitute heregulin-stimulated signaling cascades and erbB3 interactions with potential signaling partners using baculovirus-expressed proteins. For example, we will set out to determine what specific sites on erbB3 are tyrosine phosphorylated by the EGF receptor (in an EGF-dependent manner) and by neu/erbB2 (in a heregulin-dependent manner). We then will determine which heregulin-stimulated phosphorylation site on erbB3 is responsible for the formation of a stable erbB3-p85 complex,

using purified recombinant proteins. Once this has been determined, we will set out to generate erbB3 mutants that can be introduced into mammary cells via cDNA transfection approaches to determine whether these mutants influence mammary cell growth.

2.) ***Determination of the mechanisms underlying the activation of neu/erbB2 by the EGF receptor-*** *In vitro* studies have shown that while the EGF receptor is able to strongly phosphorylate the cytoplasmic domain of the neu/erbB2 protein (Guy et al., submitted; also, see "Progress Report"), this phosphorylation event does not result in an activation of the neu/erbB2 tyrosine kinase activity but rather promotes the binding of neu/erbB2 to the SH2 domains of p85 and c-src. However, recent studies in PC12 cells have suggested that when the full length neu/erbB2 protein is immunoprecipitated from PC12 cells treated with EGF, the tyrosine kinase activity of neu/erbB2 is higher than that for neu/erbB2 precipitated from untreated cells. These results suggest the possibility that the EGF-stimulated heterodimer formation between the EGF receptor and neu/erbB2 in intact cells results in the recruitment of additional cellular proteins that promote the (EGF-dependent) activation of neu/erbB2 (one possibility being c-src). We will determine whether c-src is complexed with neu/erbB2 in an EGF-dependent manner in mammary cells. We also will use insect cell expression systems to determine if c-src is capable of binding to neu/erbB2 in an EGF-dependent manner, if it is able to phosphorylate neu/erbB2 at a specific site(s), and if this phosphorylation results in the activation of the neu/erbB2 tyrosine kinase activity.

3.) ***Determination of the effects of EGF receptor-p130 interactions on c-src activity in mammary carcinoma cells-*** While searching for cytosolic targets for members of the EGF receptor subfamily of receptor tyrosine kinases, we discovered an 130 kDa protein (p130) that was strongly phosphorylated in an EGF-dependent manner. As outlined in the "Progress Report", this phospho-substrate may be related to the Crk-associated, src phospho-substrate Cas. Various lines of biochemical data suggest that p130 strongly associates with the EGF receptor (but not with neu/erbB2) through the SH3 domain of Grb2. It is tempting to speculate that the EGF-stimulated tyrosine phosphorylation of p130 results in the formation of a complex between one of the SH2 domains of p130 and c-src. We intend to verify this by immunoprecipitating p130 using an anti-phosphotyrosine antibody and determining whether c-src is present in the immunoprecipitates. We also are in the process of obtaining a full length cDNA clone for the human mammary p130 protein. Once we have obtained the full length cDNA, we will express it in insect cells for further biochemical study. If we find evidence for a p130-src interaction in mammary cells, we will use the purified recombinant proteins to identify the phosphorylation site(s) on p130 that is involved in binding to c-src. We also will use the insect cell-expressed proteins to complement the functional studies being performed *in vivo* and determine if p130-src interactions lead to an activation of src activity.

4.) ***Biochemical characterization of a heregulin-stimulated 18 kDa GTP-binding protein in human mammary cells-*** Recently, we have discovered an 18 kDa GTP-binding protein (designated p18) that is present in the nucleus of PC12 cells (see "Progress Report") and is stimulated to bind GTP upon treating the cells with NGF or heregulin. At the present time, we are attempting to obtain sufficient amounts of purified p18 for microsequencing. It will be of interest to determine how effectively this GTP-binding protein is activated in different mammary carcinoma cells upon treatment with heregulin. We will determine whether this activation is specific (i.e. if EGF also promotes the activation of p18 in mammary cells) and if this GTP-binding protein is "downstream" from erbB3 in mammary cells. The latter will be determined by examining the effects of reagents that interfere with erbB3-neu/erbB2 interactions, as they are developed (i.e. specific erbB3 antibodies or the recombinant erbB3 extracellular domain), on growth factor-stimulated binding to p18. If we find that p18 is activated in a heregulin and/or EGF-dependent manner in mammary carcinoma cells, the long term goal will be to identify the other nuclear proteins that associate with p18 and the functional outcome of this association (i.e. regulation of gene expression and/or regulation of nuclear transport, etc.).

5.) ***Establishment of an animal model system for human breast cancer-*** In the studies outlined above, we intend to characterize the molecular mechanisms that underlie heregulin-stimulated signal transduction. The goal of these studies will be to obtain molecular information

that will be useful for designing strategies for interfering with the development of mammary carcinomas. Toward that end, we intend to examine the utility of an animal model system, based on the development of spontaneous mammary carcinomas in the dog, for screening reagents that might interfere with the signaling pathways activated by heregulin (through the neu/erbB2 and erbB3 proteins). In the coming year, we will screen canine mammary carcinomas for the over-expression of the EGF receptor, neu/erbB2, and erbB3 using Northern and Western analyses. Comparisons also will be made between cell lines generated from canine mammary tumors and the different human breast cancer lines that are available in the laboratory to determine if the binding characteristics of heregulin (and EGF) and the heregulin-stimulated (and EGF-stimulated) tyrosine phosphorylation patterns are similar. As a longer term goal, any reagents that we generate from the studies outlined in Aims 1-4, above, that are capable of interfering with heregulin-stimulated neu/erbB2-erbB3 interactions (or EGF-stimulated EGF receptor-neu/erbB2 or EGF receptor-erbB3 interactions), will be examined for their effects on the growth of human tumor cells and dog tumor cells. The ultimate aim will be to identify promising reagents from these cell growth experiments that might be useful in clinical trials on dogs diagnosed with mammary carcinomas.

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MANUSCRIPTS RESULTING FROM THE RESEARCH

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PERSONNEL INVOLVED IN THE STUDIES

1.	Richard A. Cerione	Ph.D.	P.I.
2.	Daniel C. Gamett	Ph.D.	Postdoctoral Associate
3.	James Flanders	D.V.M.	Postdoctoral Fellow
4.	Kristin Wilson	B.A.	Graduate Student
5.	Jin Wu	M.D.	Postdoctoral Associate
6.	Elsa LeBars	B.A.	Veterinary Student - Technician

MEETING ABSTRACTS

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